Herpes simplex virus ICP27 regulates alternative pre-mRNA polyadenylation and splicing in a sequence-dependent manner

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The herpes simplex virus (HSV) infected cell culture polypeptide 27 (ICP27) protein is essential for virus infection of cells. Recent studies suggested that ICP27 inhibits splicing in a gene-specific manner via an unknown mechanism. Here, RNA-sequencing revealed that ICP27 not only inhibits splicing of certain introns in <1% of cellular genes, but also can promote use of alternative 5′ splice sites. In addition, ICP27 induced expression of pre-mRNAs prematurely cleaved and polyadenylated from cryptic polyadenylation signals (PAS) located in intron 1 or 2 of ∼1% of cellular genes. These previously undescribed prematurely cleaved and polyadenylated pre-mRNAs, some of which contain novel ORFs, were typically intronless, <2 Kb in length, expressed early during viral infection, and efficiently exported to cytoplasm. Sequence analysis revealed that ICP27-targeted genes are GC-rich (as are HSV genes), contain cytosine-rich sequences near the 5′ splice site, and have suboptimal splice sites in the impacted intron, suggesting that a common mechanism is shared between ICP27-mediated alternative polyadenylation and splicing. Optimization of splice site sequences or mutation of nearby cytosines eliminated ICP27-mediated splicing inhibition, and introduction of C-rich sequences to an ICP27-insensitive splicing reporter conferred this phenotype, supporting the inference that specific gene sequences confer susceptibility to ICP27. Although HSV is the first virus and ICP27 is the first viral protein shown to activate cryptic PASs in introns, we suspect that other viruses and cellular genes also encode this function.

polyadenylation | alternative splicing | DNA viruses | host–pathogen interactions | RNA 3′ polyadenylation signals

Herpes simplex virus (HSV) infected cell culture polypeptide 27 (ICP27), an immediate early (IE) gene (among those first expressed after virus enters the cells) that is required for expression of some early and late viral genes as well as for virus growth, is highly conserved between HSV-1 and -2, two closely related neurotropic herpesviruses (1). ICP27 has a role in transcriptional regulation through association with the C-terminal domain of RNA polymerase II (2, 3), forms homodimers (4, 5), interacts with U1 small nuclear ribonucleoprotein (snRNP) through its C-terminal domain, and colocalizes with U1 and U2 snRNPs (6, 7). It also interacts with splicing factors such as SRSF3, SRSF1, SRSF7, and SRSF2 (8–11), and is involved in nuclear export of some viral transcripts (12, 13). The role of ICP27 in regulating pre-mRNA splicing remains controversial. Early studies indicated that, in an in vitro pre-mRNA splicing system, ICP27 may nonspecifically inhibit host pre-mRNA splicing, impairing spliceosome assembly as a result of interaction with SR protein kinase 1 (SRPK1) through ICP27's N-terminal RGG RNA-binding motif and/or interaction with spliceosome-association protein 145 (SAP145 or SF3B2) through ICP27's C-terminal domain (8, 11). A recent communication reported that HSV-1 does not inhibit cotranscriptional splicing and proposed that previous reports of ICP27-induced splicing inhibition were artifacts, due to misinterpretation of run-on transcription (14). Indeed, splicing of only a few viral and cellular pre-mRNAs have been reported to be inhibited by ICP27 in infected cell culture. For example, splicing of alpha-globin is inhibited by ICP27 when ICP4, another viral IE gene, is present (15). ICP27 also promotes expression of the full-length glycoprotein C protein (16, 17) and a truncated form of HSV-2 ICP34.5 (18, 19), the major viral neurovirulence factor, by inhibiting splicing of these genes. ICP27 inhibits splicing of only introns 7a and 8 of promyelocytic leukemia protein (*PML*) (20). We previously reported that ICP27 inhibits ICP34.5 splicing much more efficiently than other cotransfected splicing reporter genes in a way not fully dependent on the N-terminal RGG motif, suggesting that ICP27 may inhibit splicing in a gene- or sequence-specific manner (18) that cannot be completely explained by previously proposed mechanisms (1).

Results

To further characterize the role of ICP27 in regulating host pre-mRNA processing, high-throughput RNA-sequencing (RNA-seq) data from poly-(A)-enriched RNA purified from HEK293 cells transiently transfected with or without ICP27 was analyzed. We narrowed our search from the 19,655 cellular genes with expression level ≥0.5 fragments per kilobase per million fragments mapped (fpkm) to the ∼12,000 highest-ranked genes [based on scores rating differences in expression in poly(A)-enriched RNA between ICP27-transfected and control samples] and visually examined gene expression profiles for differences in exon or intron use. ICP27 was

Significance

Although implicated, the role of herpes simplex virus (HSV) infected cell culture polypeptide 27 (ICP27) in cotranscriptional pre-mRNA processing remains poorly understood. We show that ICP27 promotes cotranscriptional cellular pre-mRNA 3′ end formation using cryptic polyadenylation signals in introns, generating hundreds of novel, intronless GC-rich cellular transcripts that resemble HSV genes. ICP27 also causes aberrant pre-mRNA splicing of some genes. ICP27-targeted genes share common features such as high GC content, cytosine-rich sequences, and suboptimal splice sites, providing an explanation for the observed target specificity of ICP27 and suggesting an overlapping mechanism for ICP27-mediated aberrant pre-mRNA splicing and polyadenylation. By specifically modifying pre-mRNA processing of HSV-like GC-rich transcripts that are likely spared by the virion host shutoff protein, ICP27 contributes to virus-induced host shutoff required for efficient viral growth.

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Data deposition: The sequence data reported in this paper have been deposited in the NCBI Sequence Read Archive (SRA), www.ncbi.nlm.nih.gov/sra (accession no. PRJNA343110).

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associated with aberrant pre-mRNA processing in >200 genes (Fig. S1 *A* and *B*). Most frequently, this association was related to premature termination of pre-mRNA because of polyadenylation from a cryptic, previously undescribed, polyadenylation signal (PAS) in intron 1 (132 genes, ~1.1%) or from a PAS in intron 2 or an alternative exon 2 in associated with retention of intron 1 (16 genes, ~0.13%). ICP27 promoted use of a cryptic downstream 5′ splice site in 12 genes. Intron retention was identified in 78 genes (~0.65%), consistent with our previous finding that ICP27 specifically inhibits the splicing only of certain genes (18). ICP27-targeted genes included genes that play roles in key cellular pathways, including transcription, DNA-damage response, stress and immunoregulation (including innate immunity), signal transduction, translation, the cell cycle, and metabolism (Table S1).

ICP27 Induces Expression of Previously Undescribed Cellular Pre-mRNAs Prematurely Cleaved and Polyadenylated from Cryptic PASs in Intron 1 or 2 or Immediately Downstream of Retained Intron 1. *PPTC7* (Fig. 1*A*), a protein phosphatase gene, and *UNK* (Fig. 1*B*), an RNA-binding zinc finger protein implicated in the control of a neuronal morphology program (21), transmembrane protein *TMEM245* (Fig. 1*C*), and the serine/threonine-protein phosphatase *PPP6C* (Fig. 1*D*) were among the 132 genes in which ICP27 induced partial retention of intron 1 with sharp decreases in read counts at a cryptic intron 1 PAS. A total of 48 (~36%) of these intronless transcripts, including the 4 examples above, contain an ORF of >110 amino acids, with predicted molecular masses ranging from 11.5 to 39.9 kDa (Table S1), and none have been previously described. Northern hybridization of total RNA prepared from cells infected with wild-type HSV

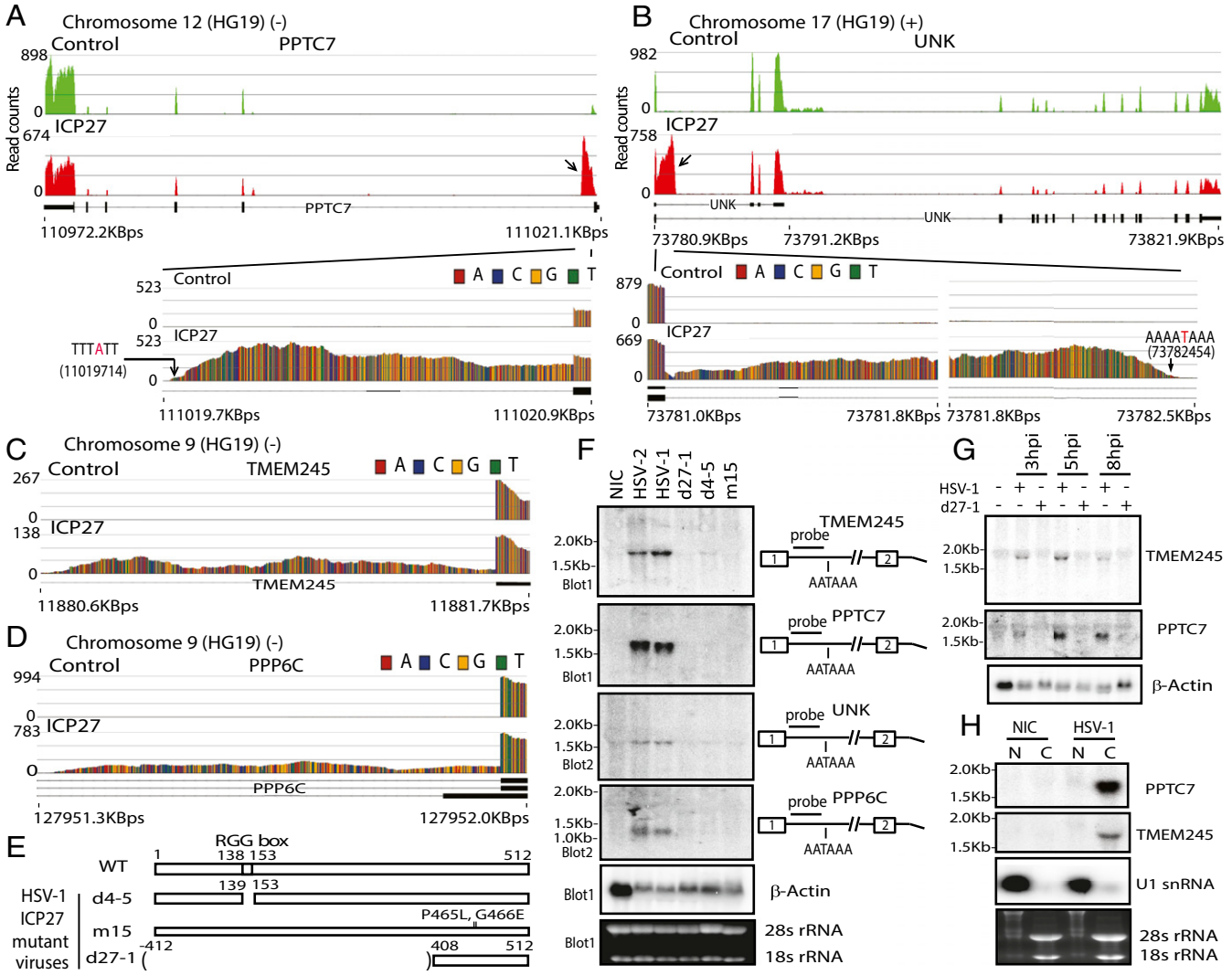

Fig. 1. HSV ICP27 activates expression of pre-mRNAs prematurely cleaved and polyadenylated from cryptic PASs in intron 1. (*A* and *B*) Read counts mapping to representative ICP27-targeted genes in poly(A)-selected RNA *PPTC7* (negative strand; *A*) and *UNK* (positive strand; *B*). Control, pFlag vector-transfected cells; ICP27, HSV-2 ICP27-transfected cells. Previously described transcript variants (thick black lines denote exons) are shown underneath. Arrows indicate significant differences in intronic read counts in ICP27-expressing cells. Blowups showing intron 1 read counts are shown below. (*C*) Blowup showing intron 1 of *TMEM245* (negative strand) read counts. (*D*) Blowup showing intron 1 of *PPP6C* (negative strand) read counts. (*E*) Domains and mutations in HSV-1 (WT) and ICP27 mutants d27-1, d4-5, and m15. (*F*) Northern hybridization of *TMEM245*, *PPTC7*, *UNK*, and *PPP6C* in HEK293 cells infected with HSV wild-type or ICP27 mutants at 8 hpi using intron-specific probes illustrated at *Right* to detect prematurely cleaved and polyadenylated pre-mRNAs. β-actin and ribosomal RNAs were used as loading controls. (*G*) ICP27-mediated prematurely cleaved and polyadenylated mRNAs are detectable during early infection. Northern hybridization for prematurely cleaved and polyadenylated *TMEM245* and *PPTC7* pre-mRNAs in HEK293 cells infected with HSV-1 KOS strain or d27-1 at 3, 5, and 8 hpi is shown. (*H*) ICP27-mediated prematurely cleaved and polyadenylated mRNAs can be efficiently exported to cytoplasm. Northern hybridization for prematurely terminated *PPTC7* pre-mRNA of cytoplasmic (C) and nuclear (N) RNA fractions from HSV-1 infected at 5 hpi or uninfected (NIC) cells is shown. The same membrane was blotted with probes for *PPTC7*, *TMEM245*, and U1 snRNA. U1 snRNA and ribosomal RNAs indicate efficiency of cytoplasmic and nuclear fraction separation.

and ICP27 mutant viruses (Fig. 1*E*), using intron sequence-specific probes, revealed that both HSV-1 and -2 activated expression of cellular genes that use intronic PASs, based on detection of bands of sizes expected from the RNA-seq analysis (Fig. 1*F*). Mutant viruses with deletion of ICP27 (d27-1) or point mutations of amino acids 465 and 466 in the ICP27 C-terminal domain (m15) did not induce expression of these alternatively polyadenylated cellular transcripts. Deletion in mutant d4-5 of the RGG/SRPK-1 binding domain, which has been shown to interact with RNA and SRPK-1 (11, 22), also sharply reduced expression of alternatively polyadenylated *PPTC7* and *PPP6C* and yielded only weak expression of alternatively polyadenylated *TMEM245* and *UNK*, suggesting that this domain also plays an important role in the processing of these prematurely cleaved and polyadenylated intronless cellular transcripts. These alternatively polyadenylated transcripts were detectable as early as 3 h postinfection (hpi) and peaked at 5 hpi (Fig. 1*G*). Intronless *PPTC7* was efficiently exported to the cytoplasm in infected cells (Fig. 1*H*).

Eukaryotic translation initiation factor 4 gamma 3 (*EIF4G3*), a translation initiation factor targeted by vaccinia virus (23), was among the 16 genes for which ICP27 induced expression of pre-mRNAs polyadenylated from a PAS in intron 2 (frequently associated with retention of intron 1) or immediately downstream of retained intron 1 of the targeted gene (Fig. S2 and Table S1). As was observed for intron 1 alternative PASs, ICP27-activated PASs in intron 2 or downstream of retained intron 1 were typically within 1.7 Kb of the transcription start site (TSS) and within 0.7 Kb of the intron 2 5′ splice site (similar to the location of ICP27-facilitated intron 1 PASs, which were typically within 1.4 Kb of the TSS and within 1 Kb of the 5′ splice site, respectively) (Fig. S3).

ICP27 Promotes Use of Cryptic 5′ Splice Sites. In 12 genes, including *ZER1* (which encodes a subunit of an E3 ubiquitin ligase complex; Fig. 2*A*) and *DESI2* (desumoylating isopeptidase 2; Fig. 2*B*), ICP27 induced partial retention of intron 1 with read counts declining abruptly not at PASs, but at potential 5′ splice site sequences. The sequences between the usual and the cryptic donor splice sequences encode alternative exons that have not been previously described. Two of these 12 genes, *LEPR* (a leptin receptor involved in fat metabolism) and *PPP1R8* (an inhibitor subunit of the major nuclear protein phosphatase-1 required for cell proliferation), are sometimes prematurely terminated at a PAS downstream of the cryptic 5′ splice site (Table S1). In all 12 genes, the impacted 5′ splice sites were within a short distance of the TSS (<1 Kb). Use of these alternative 5′ splice sites was confirmed by RT-PCR and by sequencing of HEK293 cells infected with wild-type HSV-1 and ICP27 mutants (Fig. 2*C*), showing that the cryptic *ZER1* splice site is at nucleotide 772 and that *DESI2* has two downstream cryptic splice sites, at nucleotides 979 and 991 (used at a 7:1 ratio, consistent with the read counts shown in Fig. 2*B*). Use of the cryptic 5′ splice site at nucleotide 772 changes the 5′ UTR sequence of *ZER1*, whereas use of either cryptic 5′ splice sites changes the expected coding sequence for *DESI2* (Fig. 2*C*). The ICP27 RGG domain-deleted HSV-1 mutant virus (d4-5) promoted the use of the alternative 5′ splice site in *ZER1* more efficiently than that in *DESI2*, suggesting an additional role of the RGG RNA binding domain in regulating alternative splicing of *DESI2*.

ICP27 Only Inhibits Splicing of Select Introns in Targeted Genes. In the 78 genes in which ICP27 induced retention of one or more introns, the first and last introns appeared to be most susceptible. For example, ICP27 inhibited splicing of the last intron of *POLR2A* (encoding the large subunit of RNA polymerase II) (Fig. 3*A*), introducing a frameshift and a stop codon upstream of the final exon, which encodes a C-terminal domain previously described to interact with splicing factors, polyadenylation factors, and transactivating factors and with ICP27 itself (3, 24). Through retention of the last intron, ICP27 likely reduces functional *POLR2A* expression, and contributes to ICP27-mediated alteration of *POLR2A* functions (3, 25). ICP27 also promotes retention of the first intron of *NFS1*, a cysteine desulfurase related to protein dimerization activity (Fig. 3*B*). ICP27 induced retention of four introns (16–19) near the 3′ end of *ATXN2L* (ataxin-2-like), a regulator of stress granules that is also implicated in neurodegenerative disorders (26) (Fig. 3*C*). It appears that viral infection (vs. transfection of ICP27 alone) may be more efficient in inhibiting splicing, an observation that is not explained by differences in ICP27 protein levels between transfected vs. infected cells (Fig. S4), suggesting that other viral proteins or the microenvironment created by viral infection may facilitate ICP27's function. Deletion of ICP27 (d27-1) or a two-amino-acid mutation in the C-terminal domain (m15 for the mutant virus or pM15 for the mutant plasmid) nearly abolished ICP27-mediated splicing inhibition of *NFS1* in both virus infection and transfection experiments (Fig. 3 *D* and *E*). Deletion of the N-terminal RGG/SRPK-1 binding domain in viral mutant d4-5 reduced ICP27-mediated intron retention, but not to the extent of the C-terminal (m15) mutation.

ICP27-Targeted Genes Are GC-Rich, with Suboptimal Splicing Sites and C-Rich Sequences Near the 5′ Splice Site. ICP27-mediated alternative pre-mRNA processing occurred only in relatively less abundant transcripts [based on fpkm (reads), the three most abundant ICP27-targeted mRNA transcripts in the RNA-seq experiment were ranked 283 for *MDH2*, 946 for *YWHAH*, and 1,882 for *ZNF598*). The GC content of analyzed ICP27-targeted host gene introns and exons near the impacted splice site averaged 64.5% and 68.0%, respectively, similar to that of HSV genes and much higher than that of typical human introns (46%) and exons (51%) (Fig. S5*A*; ref. 27). No example of a consensus 5′ or 3′ splice site was observed in an ICP27-targeted intron, suggesting that, although the average strength for both 5′ and 3′ splice sites was comparable to that of typical splice sites in human genes (Fig. S5*B*), ICP27-targeted splice sites are suboptimal (as are many human splicing sites). Indeed, we observed that ICP27-targeted introns are normally spliced efficiently when ICP27 is not present. Analysis using MEME GLAM2 software identified C-rich consensus sequences containing a stretch of cytosines such as CCCC(U) in exon (Fig. S6*A*) and/or intron (Fig. S6*B*) sequences near the 5′

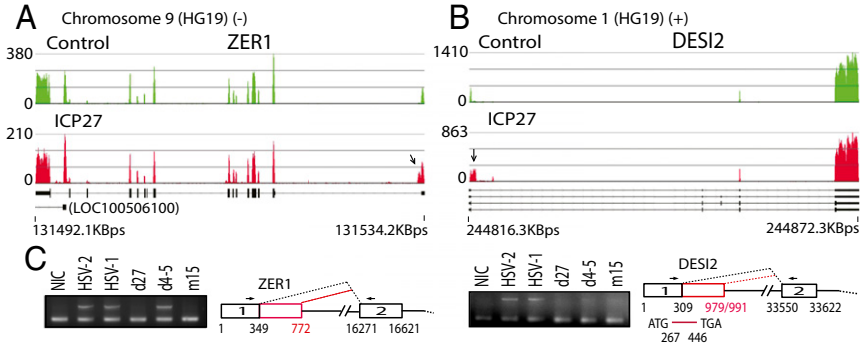

Fig. 2. ICP27 promotes use of cryptic 5′ splice sites. (*A* and *B*) Read counts mapping to *ZER1* (negative strand; *A*) and *DESI2* (positive strand; *B*). Previously described transcript variants (thick black lines denote exons) are shown below. Arrows denote differences in intron 1 between ICP27 and control-transfected cells. (*C*) RT-PCR for *ZER1* and *DESI2* of HSV-1–infected and mutant virus-infected (Fig. 1*E*) HEK293 cells. Cryptic 5′ splice sites were confirmed by sequencing of RT-PCR products and are illustrated in red. The same set of cDNAs were used for both *Left* and *Right*. NIC, noninfected control.

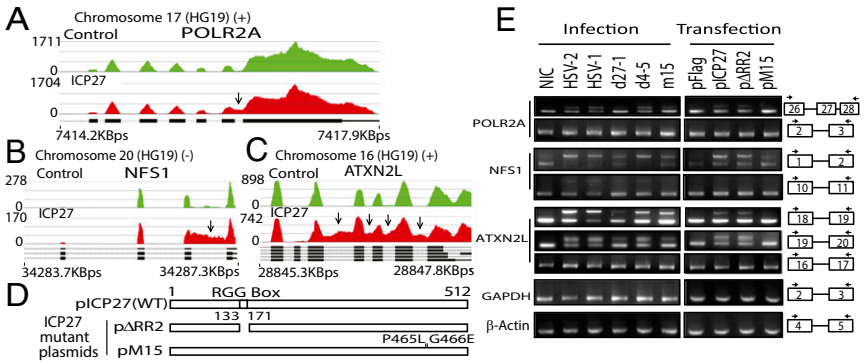

Fig. 3. ICP27-induced retention of specific introns in some host genes. (*A*–*C*) Read counts mapping to *POLR2A* (last intron retention; *A*), *NFS1* (intron 1 retention; *B*), and *ATXN2L* (multiple internal intron retention; *C*). Arrows indicate significant differences in read counts. (*D*) Schematic diagrams of inserts in HSV-2 ICP27 expression plasmids. (*E*) Effect of ICP27 mutations in HSV-1 viruses (Fig. 1*E*) and HSV-2 plasmids on intron retention of cellular genes by RT-PCR of infected (8 hpi) or transfected HEK293 cells. Arrows denote RT-PCR primers (Table S2). Exons are numbered in boxes. NIC, noninfected control.

splice site of genes for which splicing is inhibited by ICP27. In genes for which ICP27 activated intronic PAS, intronic cytosine stretches were more common (Fig. S6*D*) than were exonic cytosine stretches (Fig. S6*C*), suggesting that intronic cytosines may play a more important role in polyadenylation from intronic PAS of these transcripts in the presence of ICP27.

Splicing Inhibition Mediated by ICP27 and Cytosine-Rich Sequences Does Not Require the ICP27 N-Terminal RGG Motif. ICP27 increased the unspliced to spliced ratio of a chimeric mRNA in which the C-rich HSV-2 ICP34.5 intron was replaced with the similarly sized intron 2 from the ICP27-insensitive KSHV K8 gene (Fig. 4 *A* and *B*), whereas neither HSV-1 nor HSV-2 ICP27 significantly inhibited splicing of mutant chimeric mRNAs in which ICP34.5 exon 1 was also replaced with corresponding KSHV K8 exon 2 sequences or in which point mutations of cytosines in ICP34.5 exon 1 were introduced (Fig. 4*B*). Mutation of *ATXN2L* exon 18 C-rich sequences, whether immediately upstream of the 5′ splice site or further upstream, sharply reduced ICP27-mediated intron 18 splicing inhibition in reporter assays, whereas mutation of C-rich sequences in intron 18 or in downstream exon 19 did not (Fig. 4 *C* and *D*). Together, these results indicate that exonic C-rich sequences near the 5′ splice site are more important for ICP27-mediated splicing inhibition than intronic sequences. KSHV K8 intron 2 is normally alternatively spliced and contains suboptimal splicing sites (28, 29). Splicing in a KSHV K8 splicing reporter containing both K8 introns 1 and 2 is not inhibited by ICP27 (18). Introduction of cytosines by G to C and A to C mutations in the K8 exon 2 sequence upstream of the 5′ splice site in pK8ccct (Fig. 4*E*), greatly increased its sensitivity to ICP27-mediated splicing inhibition (Fig. 4*F*), further confirming that C-rich sequences near the 5′ splice site are involved in ICP27-mediated splicing inhibition. Additionally, an ICP27-expressing plasmid mutant with deletion of the ICP27 N-terminal RGG/SRPK-1 motif and adjacent downstream potential RNA binding sequences was nearly as efficient as wild-type ICP27 in inhibiting pK8ccct mutant splicing, further indicating that ICP27 interactions with the RNA sequence and SRPK-1 through the RGG motif are not required for ICP27-mediated specific splicing inhibition.

Suboptimal Splice Sites Contribute to ICP27-Mediated Splicing Inhibition. Replacement of the suboptimal *ATXN2L* intron 18 5′ and 3′ splice sites with consensus sequences moderately increased basal splicing efficiency in the absence of ICP27, but nearly abolished ICP27-mediated splicing inhibition (Fig. 4 *G* and *H*). This finding suggests that the suboptimal splice sites that flank all of the identified ICP27-targeted introns are required for efficient ICP27-mediated splicing inhibition, which is also in agreement with a previous report that optimization of *PML* intron 7 splicing sites abolished its sensitivity to ICP27-mediated splicing inhibition (20).

Discussion

HSV-1 and -2 ICP27 modify the pre-mRNA processing of a select group of cellular genes, leading to use of cryptic intronic PAS, use of downstream cryptic 5′ splice sites, and retention of specific introns, reducing the expression of targeted genes while increasing the protein coding diversity of these genes. Both the N-terminal RGG domain and the C-terminal domain of ICP27 are required for efficient use of intronic PAS, with the C-terminal domain being apparently more important for regulating alternative splicing. Shared sequence elements (suboptimal splice sites and C-rich sequences near the 5′ splice site) and the reduced use of a specific 5′ splice site in all cases of these ICP27-mediated effects suggest that different forms of ICP27-mediated aberrant pre-mRNA processing likely have overlapping mechanisms.

Our results confirm ICP27's role in cotranscriptional cellular pre-mRNA splicing and polyadenylation of specific transcripts, consistent with the results using splicing reporters (Figs. S7 and S8). Our findings, including identification of prematurely cleaved and polyadenylated transcripts by Northern hybridization in wild type, but not in ICP27 deletion mutant virus-infected cells, would not have been predicted by a recent report (14), which posited that ICP27 had no role in regulating cellular cotranscriptional pre-mRNA splicing or termination of cellular transcripts.

In vitro polyadenylation experiments suggested that ICP27 is involved in promoting polyadenylation from "weak" PASs of late genes, including UL44 (glycoprotein C) (30–33), suggesting that ICP27 likely directly influences both polyadenylation and splicing. ICP27's impact on polyadenylation from intronic PAS typically located within 1 kb of the 5′ splice site mirrors that recently observed when U1 snRNP's binding to the 5′ splice site was inhibited, also relieving its inhibition of CPSF binding to the downstream PAS (34–36). We hypothesize that ICP27 may thus interfere with U1 snRNP's binding to 5′ splice sites in the context of specific introns, through direct or indirect interaction with the C-rich sequences near the 5′ splice site (Fig. 4*I*). Recent crystal structure studies demonstrated that the structure of ICP27 does not have KH domains and that its C-terminal region does not fold into a potentially RNA-binding hnRNPK-like structure (4, 5). ICP27's RGG motif has been shown to directly bind RNA (37, 38) and appears to play a significant role in alternative polyadenylation and a lesser role in splicing inhibition. However, our in vitro transfection experiments and previous reports (18, 20) showing that the RGG motif is not required for ICP27-mediated splicing inhibition suggest that there may be other RNA binding sites in ICP27 or that unknown adaptor proteins are involved in recognizing the C-rich sequences near the 5′ splice site. We also note that the precise nature of the C-rich sequences important for ICP27 effects has not yet been defined.

For *LEPR* and PPR1R8 (Table S1), some RNAs were alternatively polyadenylated using intronic PAS, and others used an alternative 5′ splice site, suggesting that the relative kinetics of splicing and polyadenylation are important for alternative polyadenylation, as has been hypothesized (39). Thus, it appears that the fate of ICP27-targeted pre-mRNA is determined by the strength and proximity of splice sites, availability of C-rich sequences near the 5′ splice site, availability and proximity of an intronic PAS, the size of the intron (with larger introns more likely to show use of an alternative 5′ splice site or intronic PAS, and with smaller introns more likely to be retained), and efficiency of RNA polymerase II transcription (i.e., reduced efficiency or "pausing" of RNA

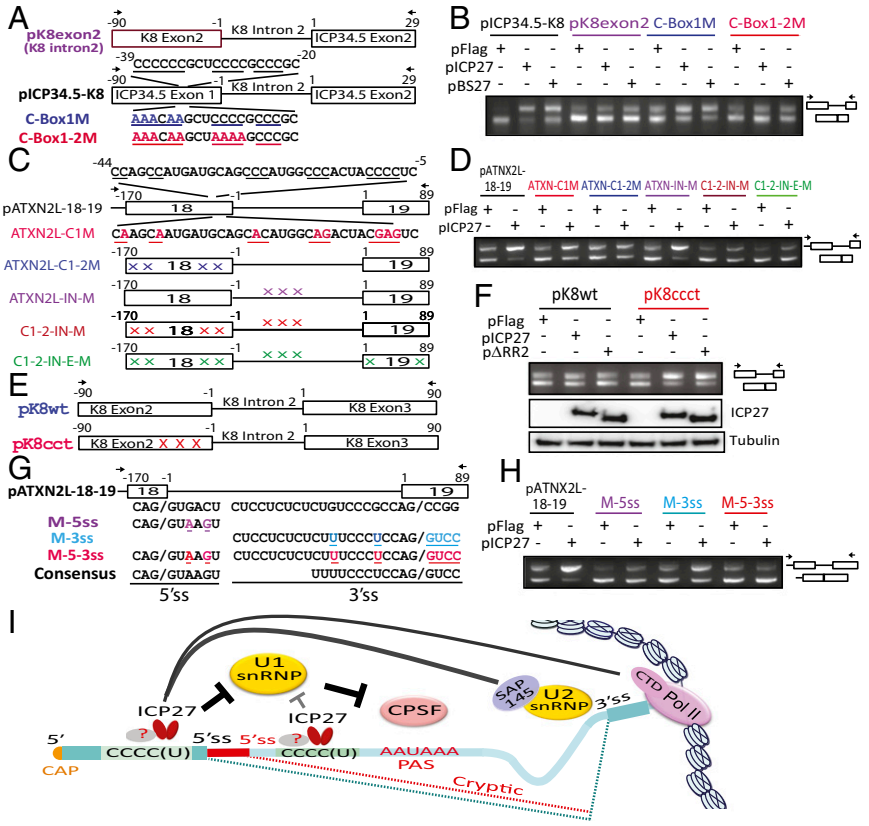

Fig. 4. Suboptimal splice sites and C-rich sequences mediate splicing inhibition by ICP27. (*A*, *C*, *E*, and *G*) Reporters used in *B*, *D*, *F*, and *H*, respectively, which show splicing analysis by RT-PCR of cells also transfected with HSV-2 ICP27 constructs (Fig. 3*D*) or HSV-1 ICP27 (pBS27). (*B*) Splicing analysis of KSHV K8/HSV-2 ICP34.5 constructs. ICP27-mediated splicing inhibition requires C-rich sequences in the 5′ exon of pICP34.5-K8. (*D*) Splicing analysis of ATXNL mutant constructs. ICP27-mediated splicing inhibition requires C-rich sequences (mutations shown by X) in the 5′ exon of pATXN2L-18-19. (*F*) Splicing analysis of KSHV K8 exon 2 mutations. Introducing C mutations (at X) in the 5′ exon of an ICP27-insensitive reporter enhances splicing inhibition by ICP27, independently of the N-terminal RGG motif. (*H*) Splicing analysis of *ATXN2L* mutants. Optimizing *ATXN2L* intron 18 splice sites abolishes ICP27-mediated splicing inhibition. (*I*) Proposed mechanism of ICP27-mediated cotranscriptional aberrant pre-mRNA processing. ICP27 (known to interact with U1 snRNP, U2 snRNP and the Pol II CTD) may prevent U1 binding to 5′ splice sites near C-rich sequences, causing inefficient spliceosome assembly and relief of U1-snRNP-mediated inhibition of CPSF binding to intronic PAS. CPSF, cleavage and polyadenylation specificity factor, Pol II CTD, RNA polymerase II C-terminal domain. Mutations are colored; arrows denote RT-PCR primers.

polymerase II at the TSS or at suboptimal 3′ splice sites favors alternative polyadenylation). Because ICP27 appears to target less abundant transcripts and expression of many genes is tissue-specific, it is possible that ICP27 has tissue-specific targets.

ICP27-induced aberrant pre-mRNA processing likely leads to reduced expression of many affected cellular genes and alteration in the UTR sequence of other cellular transcripts that may alter mRNA stability. ICP27-induced aberrant pre-mRNA processing likely also leads to expression of novel truncated or frameshifted host cell proteins, expanding the genomic material available to the virus. Although aberrant pre-mRNAs containing premature termination codons (PTCs) are often subjected to degradation via nonsense-mediated decay (NMD) (40), at least some ICP27-mediated aberrant pre-mRNAs contain PTCs are able to escape NMD and express proteins, including full-length glycoprotein C and HSV-2 ICP34.5β (16, 18). It thus seems likely that at least some of these host transcripts can also express novel proteins. Recent studies suggested that the virion host shutoff-RNase (vhs) protein, previously thought to nonspecifically degrade host and viral mRNAs, more selectively targets specific host mRNAs, but not GC-rich viral mRNAs (41, 42). Because the GC content of ICP27-targeted genes is similar to that of HSV genes, they also likely escape selective degradation by vhs. Thus, by specifically modifying pre-mRNA processing of HSV-like GC-rich transcripts that are likely spared by the virion host shutoff protein, ICP27 contributes to virus-induced host shutoff required for efficient viral growth.

ICP27 affects pre-mRNA processing of >200 genes in ICP27-transfected cells involved in important cellular pathways, implying a broad program of ICP27-mediated cellular modification to favor the virus, and helping to explain the observation that ICP27 expression is toxic to the cell and is both required for efficient virus growth and for severe symptoms (43–45). Of the affected genes, >30, including *PML*, *STING*, *TRAF6*, *PPP6C*, *MAP3K7*, *FBXW11*, *IFNAR2*, *NFKB1*, *RELA*, and *CREBP*, are related to innate immunity pathways, which is consistent with ICP27's known role in regulating innate immunity (46–49). Although it would not be practical to separately examine these effects in each of these genes, it seems likely that the combined effect of these alterations exceeds that of any one. It has been reported that ICP27-induced intron retention in *PML* appears to alter viral growth (20), that alternative splicing in viral gC plays an important role in viral immune evasion by regulating the relative expression of full-length and secreted forms of gC (16), and that ICP27 alters viral neurovirulence through inhibition of HSV-2 ICP34.5 splicing (18).

Although HSV is the first virus and ICP27 is the first viral or cellular protein shown to promote expression of pre-mRNAs prematurely cleaved and polyadenylated from intronic PAS, we suspect that other viruses or unidentified cellular genes also encode this function. Further investigation will likely yield insight both into mechanisms of viral pathogenesis, potentially leading to identification of new targets for antiviral strategies, and into the mechanisms by which the cell itself controls alternative polyadenylation and splicing of selected genes. ICP27 could also

potentially be used as a template for future design of proteins that influence cellular gene expression in this manner.

Methods

HEK293 cells were transfected with pICP27 or pFlag vector by using Lipofectamine 2000. More than 95% transfection efficiency was achieved, as determined by fluorescence microscopy of cells transfected with the same amount of pEGFP-C1 (Clontech). At 48 h after transfection, total RNAs were purified with the All-Prep DNA/RNA Kit (Qiagen). cDNA libraries were prepared from polyadenylated RNA by using the Truseq RNA Sample Kit V2 (Illumina) and were sequenced on the HiSeq 2500 according to the manufacturer's instructions (Illumina). The two samples shared a single sequencer lane. The resulting paired-end sequencing data were first aligned to the HG19 reference human genome by using Partek Flow and then further analyzed by using the Partek Genomics Suite according to the software instructions. A total of 19,655 genes were selected after applying expression-level filters (≥0.5 fpkm) for both the control (pFlag vector-transfected sample) and the ICP27 (ICP27-transfected sample) from a total of 45,000 identified genes. Genes were ranked by scores of differential expression. The expression profile of each of the first 12,000 genes for both control and ICP27 samples was visually examined. Other methods are described in *SI Materials and Methods*.

Supporting information includes *SI Materials and Methods*, Figs. S1–S8, and Tables S1 and S2.

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